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Estimation of sulphydryls in milk with n-ethyl maleimide*)

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22 **Sulfhydryle-Bestimmung** (Milch).

„Milchwissenschaft" **22.** 682—685 (1967)

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In the processing of milk changes that take place in the proteins and other constituents in milk present a number of problems particularly in connection with the preparation of various forms of concentrated and dried milk products. Heating produces distinct flavours and increased oxidative stability in milk and its products and at the same time is accompanied by the „Unmasking of the sulphydryl groups" in the whey fraction of milk proteins as observed by several workers like Gould and Sommer (13) Zweig and Block (28) Hutton and Patton (16). Roes (24) reported that it affects the heat stability of milk. Kanan and Jenness (18) reported its important role in the rate of clotting of milk by rennet. Consequently efforts are being made to quantitatively estimate the sulphydryl groups in milk and its products. The widely used amperometric titration method has presented difficulties in its application to milk as has been reported by Burton (6), Yoshino, Wilson and Herreid (27). The thiamine disulphide method of Harland and Ashworth (15) is somewhat elaborate and time consuming. Several techniques based on reagents specific to sulphydryl groups, namely P-chloromercuribenzoate (5), (10), and 5,5 dithiobis (2-nitrobenzoate (9) were found unsuitable for use with milk. Chapman and McFarlane (7) published a method for determining reducing substances in milk and milk products. The reducing capacity of milk and its products was measured by Crowe, Jenness and Coulter (8). This method was adopted from the procedure used by Anson (2), (3) Mirsky (20) and Mirsky and Anson (21) for determining sulphydryl groups in proteins. A new method for determining the free and masked sulphydryl groups in heated milk and milk products has been reported by Lyster (19). The procedure was based on the use of specific sulphydryl reagent, p-chloro-mercuribenzoate and utilising another specific reagent, 5-5 : dithiobis (2-nitrobenzoate) as an indicator producing yellow colours which are matched with the standard glasses in the Lovibond comparator. Since the first report of quantitative reaction of N-Ethylmaleimide (NEM) by Friedmann et al (11), (12), several workers like Benesche et al (4) and Price et al (23) have applied this type of reaction to the detection of various sulphydryl compounds on chromatograms. Tsou et al (26) have applied the same technique on tissue cultures. Friedmann (11) and Gregory (14) have reported that NEM reacts rapidly with the sulphydryl compounds at neutral pH. The rate of reaction of equimolar amounts of NEM and reduced glutathione have been followed by Gregory (14) spectrophotometrically by a decrease in the absorption of the former at 300 mμ, but the reaction does not go to completion. Roberts and Rouser (25) showed that the change in the absorbance at 300 mμ is proportional to the concentration of cysteine and glutathione when NEM is present in excess. The decrease in absorption of this compound at 300 mμ has been used as an assay method for sulphydryl groups. Sulphydryl solutions having concentrations as low as 0.0001 M can be assayed. The same author has successfully used to determine sulphydryl concentrations in the tissue extracts and whole blood.

In the present study NEM has been used for the extimation of free sulphydryls in milk at 300 mμ.

Experimental

Raw Materials

Raw milk samples of cow and buffalo were obtained from the Institutes farm and the pasteurised milk from the dairy. The reagents used were of AR grade. Glass distilled water was used in the present study. The readings of the optical densities were taken in the Beckmann DU spectrophotometer at 300 mμ.

Results

The following trials were carried out to standardise the conditions for estimating —SH groups in milk.

I. Effect of the concentration of NEM on the optical density :

An aqueous solution containing 23.0—110 mg/100 ml of N-ethylmaleimide was prepared and a 5 ml aliquot of this was made upto 20 ml with water and the optical densities were read at 300 mμ. Effect of concentration of NEM on the optical density is presented in Table I.

Table No. I. Effect of N-Ethylmalemide concentration on the optical density of its solution

S. No.	Concentration of N-Ethylmaleimide standard solution mg. per 100 ml.	Concentration of N-Ethylmaleimide solution in mg. per 20 ml.	Optical density at 300 mμ.
1.	23.200	1.661	0.390
2.	33.500	1.675	0.420
3.	42.800	2.014	0.500
4.	49.760	2·488	0.600
5.	79.600	3.980	0.875
6.	81.580	4.079	0.950
7.	105.760	5.238	1.300
8.	110.000	5.500	—

It was observed from the table that a concentration of about 50 mg/100 ml is within the satisfactory working range.

II. Effect of pH on the rate of reaction between N-Ethylmaleimide and sulphydryl groups :

Having adjusted the optimum concentration of NEM to about 50 mg/100 ml, studies were undertaken on the optimum pH at which the maximum difference in optical densities between NEM and NEM containing cysteine HCl.

The pH range studied was between 5.0 to 8.0 using 0.2 M phosphate buffer. The buffer solutions at different pH's was made by mixing KH_2PO_4 and NaOH as described by the AOAC (22). Five ml of cysteine HCl (15 mg per 100 ml in glass distilled water) was added to 5 ml of NEM solution (in glass distilled water 50 mg/100 ml) and the volume made upto 20 ml with 0.2 M phosphate buffer of the desired pH. The optical densities were

measured at 300 mμ in a Beckmann DU spectrophotometer against reagent blanks. The results are presented in Table II.

Table No. II. Effect of pH on the rate of reaction between N-Ethylmaleimide and sulphydryl group

S. No.	pH (0.2 M phosphate buffer) (1)	Optical density of N-Ethylmaleimide solution (2)	Optical density at 300 mμ of N-Ethylmaleimide solution containing sulphydryl groups (3)	Differences in optical density due to blocking of N-Ethylmaleimide by sulphydryl groups (3—2)
1.	5.8	0.480	0.470	0.010
2.	6.0	0.480	0.460	0.020
3.	6.2	0.480	0.450	0.030
4.	6.4	0.480	0.430	0.050
5.	6.6	0.480	0.400	0.080
6.	6.8	0.480	0.385	0.095
7.	7.0	0.480	0.375	0.105
8.	7.2	0.480	0.395	0.085
9.	7.4	0.460	0.380	0.080
10.	7.6	0.460	0.385	0.065
11.	7.8	0.440	0.385	0.055
12.	8.0	0.440	0.350	0.050

It was observed that a neutral pH appears to give maximum differences in optical densities of NEM solutions and NEM solutions containing cysteine HCl.

III. Effect of temperature on the reaction between N-Ethylmaleimide and cysteine HCl :

For this study 5 ml of standard cysteine HCl solution (4 mg/100 ml) in distilled water was added to 5 ml of N-Ethyl maleimide (50 mg/100 ml) followed by 5 ml of 0.2 M phosphate buffer of pH 6.0 and then made up to 20 ml with buffer solution. The solution was subjected to heat treatment for 30 minutes from room temperature (about 30°C) to 90°C. Blanks were prepared in the usual manner.

The data is presented in Table III.

There appears to be no effect of temperature on the reaction between NEM and —SH groups.

Table No. III. Effect of temperature on the reaction between N-Ethylmaleimide and Cysteine hydrochloride

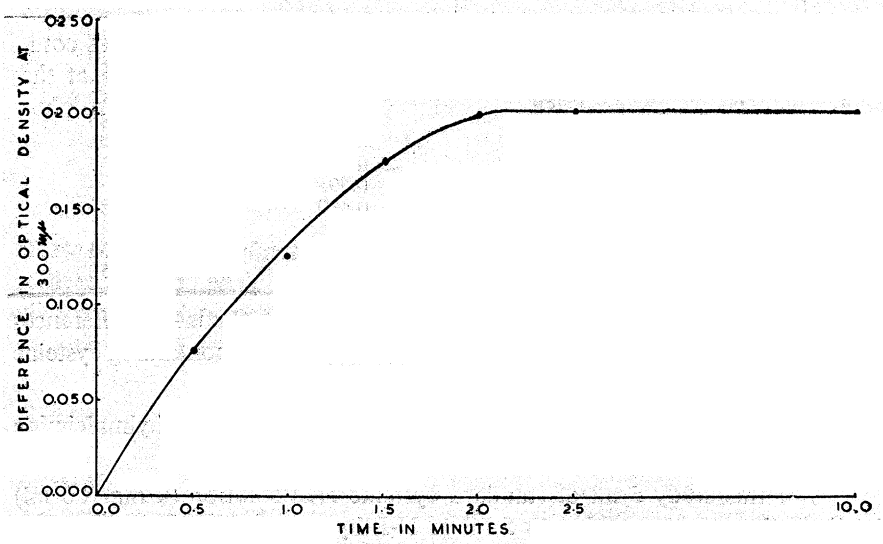
S. No.	No. of samples (1)	Time & temperature of heating (2)	Optical density of N-Ethyl-maleimide solution at 300 mμ (3)	Optical density of N-Ethyl-maleimide at 300 mμ (4)	Difference (5)
1.	5	Room temperature about 30°C	0.480	0.460	0.020
2.	5	50°C upto ½ hr.	0.460	0.440	0.020
3.	5	60°C upto ½ hr.	0.470	0.450	0.020
4.	5	70°C upto ½ hr.	0.480	0.450	0.030
5.	5	80°C upto ½ hr.	0.480	0.460	0.020
6.	5	90°C upto ½ hr.	0.480	0.460	0.020

IV. Effect of time for completion of reaction between N-Ethylmaleimide and sulphydryl groups :

For the above study the solutions were mixed as indicated earlier under temperature studies and the reaction between cysteine HCl and N-Ethylmaleimide was carried out at the room temperature and the spectrophotometric readings were taken at definite intervals of time and the data is presented in Figure 1.

Fig. 1

Effect of time on the difference in optical density of the NEM and NEM solution containing sulphydryl groups



It was observed that for completion of reaction a minimum time of two minutes was required. By keeping the solution at room temperature for one hour there was practically no change in the optical densities. Hence it would be desirable to take the reading within one hour.

V. Effect of deproteinizing agents for milk on optical densities of N-Ethylmaleimide solution and Cysteine HCl :

The following milk deproteinizing agents were selected for the study :

(a) Sodium acetate and acetic acid (17) ;
(b) Acetic Acid (17);
(c) Sodium citrate and citric acid (22);
(d) Trichloroacetic acid 10%;
(e) Metaphosphoric acid 6% ;
(f) Phosphotungstic acid 10%;
(g) Phosphomolybdic acid 10%.

It was observed that sodium acetate-acetic acid and trichloroacetic acid gave minimum interference in the maximum optical density differences obtained as a result of the reaction between NEM solution and NEM solution containing—SH groups at 300 $m\mu$ as compared to the other deproteinsing agents. However, with trichloroacetic acid the rate of preparation

of the aliquot was much faster as compared with sodium acetate and acetic acid. Hence the use of trichloroacetic acid in the present investigation was preferred.

VI. Studies on the order of addition of reagents on the reaction between N-Ethylmaleimide and sulphydryl groups :

In the earlier stages there were considerable difficulties in getting the reaction completed between NEM and —SH groups. Study was, therefore, undertaken to see if the order in which the reagents are added has any effect on the rate of reaction. If the TCA was added first the pH of the reaction mixture drops to about 4.6 (isolectric point of casein) and on addition of NEM the reaction proceeds very slowly. However, if the NEM solution was added first the reaction between the sulphydryls and NEM was completed within two minutes. Later when TCA was added the pH of the final reaction mixture dropped to around 4.6. This does not affect the sulphur bridge between the sulphydryls and NEM, and probably explains for the maximum differences in optimal densities obtained.

VII. Stabilities of NEM and cysteine HCl solution :

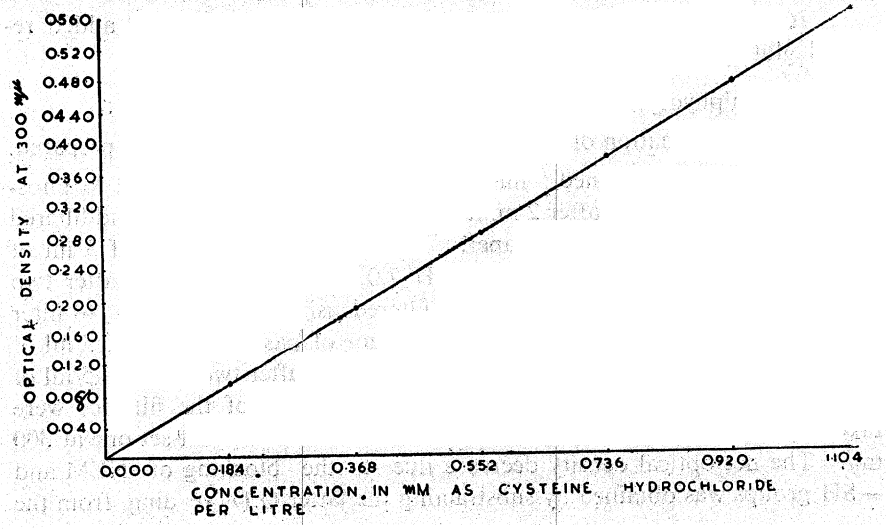
It has been observed that NEM reaction with cysteine HCl was not stable for long when both were prepared in aqueous medium. The optical densities dropped down rapidly and practically dropped to zero in 24 hours. By preparing the above reagents in 0.2 M phosphate buffer of pH 7.0 the reaction between NEM and cysteine HCl was stable even upto 24 hours at 4°C. The NEM stock solution can be kept for 3 days without much deterioration.

VIII. Preparation of standard curve :

Cysteine HCl was used at a concentration of 0—0.920 mM/litre. The conditions like pH, time and temperature were the optimum required, viz., pH 7.0 ; time 10 minutes and room temperature. The optical densities were read at 300 mμ. The standard curve obtained is presented in Figure 2.

Fig. 2

Concentration in mM as cysteine hydrochloride/litre standard curve using NEM method



IX. Recovery of added cysteine HCl and reduced glutathione from milk :

Studies were undertaken for recovery of added reduced glutathione and cysteine HCl from cow and buffalo milk (raw as well as pasteurised) using NEM.

The data are presented in Table IV.

Table No. IV. Recovery of added cysteine hydrochloride and reduced glutathione from milk by N-Ethylmaleimide

Type of milk & Sample No.	Amount of cysteine hydrochloride added to milk (mg/100 ml)	Amount of cysteine hydrochloride recovered from milk (mg/100 ml)	Percentage recovery	Amount of reduced glutathione added to milk (mg/100 ml)	Amount of reduced glutathione recovered from milk	Percentage recovery
Cow (raw)						
1.	1.5703	1.5420	98.20	1.2635	1.2016	95.10
2.	1.8985	1.8700	98.50	3.7905	3.6692	96.80
3.	3.1405	3.0433	98.03	13.2000	12.5994	95.45
4.	9.4925	9.4921	99.99	16.5000	16.4670	99.80
5.	11.3910	11.2395	98.67	19.8000	19.7999	99.99
Buffalo (raw)						
1.	2.7060	2.7060	100.00	2.8145	2.7700	98.42
2.	3.1375	3.0760	98.04	5.6290	5.3892	95.74
3.	3.6080	3.5546	98.52	8.4435	8.3016	98.32
4.	3.7390	3.7390	100.00	14.0725	13.9599	99.20
5.	7.8438	7.7418	98.70	16.8870	16.8870	100.00
Cow (pasteurised)						
1.	2.3895	2.3895	100.00	1.8985	1.8685	98.04
2.	4.5338	4.4385	97.90	2.5124	2.5124	100.00
3.	5.7750	5.4897	95.06	3.1375	3.0765	98.05
4.	7.1685	6.9248	96.60	5.7750	5.6529	97.88
5.	9.0675	8.7093	96.05	11.3910	11.2201	98.49
Buffalo (pasteurised)						
1.	3.7970	3.6910	97.21	3.7905	3.6814	97.12
2.	5.6955	5.6955	100.00	7.1685	7.0125	97.85
3.	7.5900	7.4989	98.80	13.2000	13.1845	98.88
4.	9.4125	9.2901	98.70	14.0725	13.9102	98.84

It was observed that there was over 95 per cent recovery of added reduced glutathione and systeine HCl.

X. Free sulphydryl content of pasteurised cow and buffalo milks :

For the estimation of free sulphydryls in milk three sets were prepared.

The first set contained 5 ml of pasteurised milk, 10 ml of 0.2 M phosphate buffer pH 7.0 and after 2 mintues 5 ml. of TCA was added and filtered using Whatman No. 40 filter paper. The second set consisted of 5 ml of raw milk, 5 ml of phosphate buffer pH 7.0, 5 ml of NEM and after two minutes, 5 ml of TCA was added and filtered using Whatman No. 40 filter paper. The third set was prepared with 5 ml of pasteurised milk, 5 ml of 0.2 M phosphate buffer pH 7.0, 5 ml of NEM and after two minutes 5 ml of TCA was added and filtered. The optical densities of the filtrates were taken by reading set two against set one and set three against set one at 300 mμ. The net optical density decrease due to the blocking of NEM and —SH groups was obtained by substracting the latter O.D. reading from the

former and taking reading on the standard curve making necessary corrections for dilutions.

Table No. V. Free sulphydryl content of pasteurised milk

(mM—sulphydryl per litre)

Sample No.	Cow milk	Buffalo milk
1	0.193	0.073
2	0.184	0.129
3	0·220	0.092
4	0·175	0.092
5	0.193	0.055
6	0.181	0·073
7	0·177	0.092
8	0.211	0.123
9	0.195	0.183
10	0.180	0.061
11	0.201	0.055
12	0.188	0.092
13	0.194	0.073
14	1.181	0.129
15	0.215	0.147
16	0.203	—
17	0.197	—
18	0.176	—
19	0.220	—
20	0.184	—
Maximum	0.220 mM/ilter	0.183 mM/litre
Minimum	0.175 „ „	0.055 „ „
Average	0.193 „ „	0.098 „ „

Milk was pasteurised by the H.T.S.T. (165°F for 15 seconds).

The data for the free sulphydryl content of a cow and buffalo pasteurised milk are presented in Table V.

Discussion

The interaction of the sulphydryl groups with N-Ethylmaleimide in aqueous solution at neutral pH has been developed for milk system. The optimum conditions like temperature has been observed to be that of room temperature. Similar conditions were observed by Roberts and Rouser (25) for the quantitative esitmation of free sulphydryls. The same authors used a pH of 6.0 at room temperature. Alexander (17) has developed NEM method for the estimation of thiols and the same author has reported that it was possible to estimate 0·0001 M sulphydryls. In the present investigation the readings were taken at 300 mμ in a Beckman DU spectrophotometer as the same had maximum absorbance and the same was also observed by Roberts and Rouser (25) and Alexander (11). In the present study the reaction was carried out in 0·2 M phosphate buffer at pH 7.0 instead of 0.1 M phosphate buffer suggested by Roberts and Rouser (25) as it was observed that 0.2 M phosphate buffer was convenient for the milk system. It was observed in the present study that the NEM concentration should be in excess of the —SH concentration agreeing with Roberts and Rouser (25).

The reaction was faster at neutral pH and the same was also observed by Gregory (14).

TCA and acetic acid and sodium acetate mixture appeared to be the best deproteinising agents for milk. However, with TCA there was clearer serum and the precipitation was much faster. The addition of NEM before the addition of TCA to precipitate the proteins appears to be very important as NEM first combines with the sulphydryl groups and the addition of TCA to remove the proteins does not affect the data as observed in the present study. There was more than 95% recovery of added cysteine HCl and reduced glutathione. The reaction was complete between NEM and —SH groups in about 2 minutes and the readings should be taken within one hour in the spectrophotometer. However, if the above serum is kept in the refrigerator at 0—4°C the optical densities have been observed to be stable upto 24 hours.

The range of free sulphydryl content of cow pasteurised milk was 0.175 mM to 0.220 mM with an average of 1.193 mM/litre. The free sulphydryl content of buffalo milk has been observed to be varying from 0.055 to 0.183 with an average of 0.098 mM/litre.

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Zusammenfassung

NARANG, A. S., SINGH, J., RAO, R. V., und BHALERAO, V. R. : **Bestimmung freier Sulfhydryle mit N-Aethylmaleimid.** *„Milchissenschaft"* **22.** *682—685 (1967).*

22 Sulfhydryle-*B*estimmung (Milch).

Es wird eine Methode zur Bestimmung freier Sulf-hydrylgruppen (—SH) in erhitzter Milch unter Verwendung von N-Aethylmalemid mit Hilfe eines UV-Spektralfotometers beschrieben. Die optischen Bedingungen, die fur die Bestimmung der freien Sulfhydrylgruppen in Milch erforderlich sind, werden diskutiert.

Der Gehalt an freien Sulfhydrylgruppen in pasteurisierter (H.T.S.T.) Kuh- und Buffelmilch wird bestimmt.

Pasteurisierte Buffelmilch weist einen niedrigeren Gehalt an freien Sulfhydrylgruppen auf als Kuhmilch.

Dok.-Ref.

NARANG, A. S., SINGH, J., RAO, R. V., und BHALERAO, V. R. : **Estimation of free sulphydryls in milk with N-Ethylmaleimide.** *„Milchwissenschaft"* **22.** *682—685 (1967).*

22 Sulphydryls determination (milk).

A method has been described for the estimation of „Free sulphydryl groups(—SH)" in heated milk using N-Ethylmaleimide in the UV range of a spectrophotometer. The optical conditions required for the estmation of the free sulphydryl groups in the milk using N-Ethylmaleimide has been discussed.

The free sulphydryl content of pasteurised (H.T.S.T.) cow and buffalo milk has been estimated. The buffalo pasteurised milk had lower free sulphydryl content as compared to cow milk.

NARANG, A. S., SINGH, J., RAO, R. V., et BHALERAO, V. R. : **Determination des sulfhydryles libre au moyen du N éthylmaleimide.** *„Milchwissenschaft"* **22.** *682—685 (1967).*

22 Sulfhydryles (détermination en lait).

NARANG, A. S., SINGH, J., RAO, R. V., y BHALERAO, V. R. : **Determinación de los grupos —SH libres mediante N etilmaleiuida.** *„Milchwissenschaft"* **22.** *682—685 (1967).*

22 Grupos -SH (determinación en leche).